

# Bioconversion of methane to C-4 carboxylic acids using carbon flux through acetyl-CoA in engineered *Methylobacterium buryatense* 5GB1C

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## ABSTRACT

Methane, the primary component of natural gas, is the second most abundant greenhouse gas (GHG) and contributes significantly to climate change. The conversion of methane to industrial platform chemicals provides an attractive opportunity to decrease GHG emissions and utilize this inexpensive and abundantly available gas as a carbon feedstock. While technologies exist for chemical conversion of methane to liquid fuels, the technical complexity of these processes mandate high capital expenditure, large-scale commercial facilities to leverage economies of scale that cannot be efficiently scaled down. Alternatively, bioconversion technologies capable of efficient small-scale operation with high carbon and energy efficiency can enable deployment at remote methane resources inaccessible to current chemical technologies. Aerobic obligate methanotrophs, specifically *Methylobacterium buryatense* 5GB1, have recently garnered increased research interest for development of such bio-technologies. In this study, we demonstrate production of C-4 carboxylic acids non-native to the host, specifically crotonic and butyric acids, from methane in an engineered *M. buryatense* 5GB1C by diversion of carbon flux through the acetyl-CoA node of central 'sugar' linked metabolic pathways using reverse  $\beta$ -oxidation pathway genes. The synthesis of short chain carboxylic acids through the acetyl-CoA node demonstrates the potential for engineering *M. buryatense* 5GB1 as a platform for bioconversion of methane to a number of value added industrial chemicals, and presents new opportunities for further diversifying the products obtainable from methane as the feedstock.

## 1. Introduction

Methane, a potent greenhouse gas (GHG) with 84 times more Global Warming Potential compared to carbon dioxide over a 20 year period (Greenhouse Gas Emissions, 2017), is also an energy rich, inexpensive and abundantly available carbon source. It is the primary component of bio-gas and natural gas, significant fractions of which are wasted due to flaring at production sites. For example, in 2012 3.5% of the world's natural gas supply was flared (Tollefson, 2016) and the amount of natural gas flared in the United States has more than doubled from 120 billion cubic feet in 2005 to 278 billion cubic feet in 2015 (U.S. Natural Gas Vented and Flared, 2017). The flaring of natural gas is driven by the economics of natural gas recovery, which require a given rate of methane production to justify infrastructure expenditures needed to gather, process, and transport gaseous methane (Fei et al., 2014; Clomburg et al., 2017). Furthermore, the lack of gas-to-liquid (GTL) conversion technologies that can be efficiently deployed at the small-scale results in stranded gas at remote and marginal natural gas drilling

sites (Haynes and Gonzalez, 2014; Strong et al., 2016). Existing chemical GTL processes are technologically complex, requiring multiple-unit operations and resulting in high capital cost facilities that cannot be effectively scaled down (Haynes and Gonzalez, 2014). Thus, the development of bio-GTL conversion technologies that are readily deployable at small-scale, remote natural gas drilling sites (or other distributed sources of methane emissions such as landfills or agricultural biogas) can enable recovery of abundantly available carbon and potentially allow on-site bio-conversion of otherwise wasted or flared methane into useful industrial platform chemicals and fuels (Fei et al., 2014; Clomburg et al., 2017). In addition, lowering natural gas prices and global efforts to reduce greenhouse gases such as methane are other key drivers fueling development of bio-GTL technologies.

Methanotrophs, organisms that use methane as the sole carbon and energy source (Hanson and Hanson, 1996; Whittenbury et al., 1970; Anthony, 1982), can be envisioned as promising industrial biocatalysts for bioconversion of methane to commercially relevant chemicals and fuels (Strong et al., 2016, 2015; Kalyuzhna et al., 2015; Conrado and

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Gonzalez, 2014). These organisms have been used for over 30 years for the production of products such as single cell protein (Norferm, Calysta: [www.calysta.com](http://www.calysta.com)), animal feed components (Bothe et al., 2002; Ye and Kelly, 2012), polyhydroxybutyrates (Criddle et al., 2013), methanol (Mehta et al., 1991), carotenoids, such as astaxanthin, (Ye and Kelly, 2012) and bio-diesel (Fei et al., 2014), as well as for environmental bio-engineering applications (Hwang et al., 2007). However, these efforts have met minimal commercial success, in part hindered by the lack of genetic tools available for engineering methanotrophs, slowing progress and limiting the range of products.

Recently, *Methylobacterium buryatense* 5GB1 has been identified as a genetically tractable, aerobic and obligate methanotroph (Kaluzhnaya et al., 2001; Puri et al., 2015) that offers tremendous potential to become an industrial host for methane bio-conversion due to the availability of a variety of genetic manipulations tools (Kalyuzhnaya et al., 2015; Puri et al., 2015; Yan et al., 2016) and recent advances in understanding of its metabolic pathways such as fatty acid biosynthesis and TCA cycle (Demidenko et al., 2016; Fu et al., 2017). As a Group I Gammaproteobacterium, *M. buryatense* assimilates carbon from methane at the formaldehyde level via the Ribulose Monophosphate (RuMP) pathway (Kaluzhnaya et al., 2001), generating fructose-6-phosphate that enters 'sugar'-linked core metabolic pathways to generate central metabolites such as phosphoenolpyruvate, pyruvate and acetyl-CoA. The presence of the Embden-Meyerhof-Parnas (EMP), Entner-Doudoroff (EDD) and Pentose Phosphate (PPP) pathways (Kalyuzhnaya et al., 2015, 2013), in addition to the recently discovered Phosphoketolase (PKT) pathway (Henard et al., 2015) in this host means that metabolic engineering strategies established in industrial strains such *Escherichia coli* and *Saccharomyces cerevisiae* can be implemented in *M. buryatense* as 'drop-ins' to generate a variety of products from central metabolites. Furthermore, the availability of a draft genome sequence (Khmelenina et al., 2013), a validated genome scale metabolic model (de la Torre et al., 2015), and bio-reactor performance parameters (Gilman et al., 2015) can enable rapid implementation and evaluation of metabolic engineering strategies for target product synthesis. These factors, combined with its tolerance to wide-ranging growth conditions and high resistance to contamination along with relatively rapid doubling time (2–3 h) compared to other methanotrophs (Kaluzhnaya et al., 2001), make it a promising host for the development of industrial processes.

In addition, a recent study demonstrated the production of 0.8 g/L L-lactate from methane by *M. buryatense* 5GB1 through over-expressing a recombinant lactate dehydrogenase (*ldh*) from *Lactobacillus helveticus* (Henard et al., 2016). This effort demonstrated applicability of this strain as a host for metabolic engineering approaches, however product synthesis was limited to utilizing carbon flux through the pyruvate node of central metabolism. To date, non-native product formation from acetyl-CoA, a node through which an array of promising fuel and chemical molecules can be synthesized, in *M. buryatense* or other Group I or Group II methanotrophs has proved challenging. In this study, we report the diversion of carbon from the acetyl-CoA node to product synthesis through a heterologous pathway in *M. buryatense* 5GB1C using methane as the sole carbon and energy source. Specifically, we demonstrate production of crotonic acid in *M. buryatense* 5GB1C by functional expression of a modified version of reverse  $\beta$ -oxidation pathway previously demonstrated in *E. coli* (Kim et al., 2016; Clomburg et al., 2012). This unsaturated C-4 carboxylic acid is utilized in many industrial applications, with its main use as a monomer for the synthesis of copolymers with a broad range of application such as paints, adhesives, coatings, ceramics, and agrochemicals (Blumenstein et al., 2015). By over-expressing genes from various biological sources, we obtained up to 70 mg/L of crotonic acid, in addition to the unexpected formation of 40 mg/L butyric acid, from methane in *M. buryatense* 5GB1C. In summary, we report a proof-of-concept of production of industrial platform chemicals from methane by utilizing the carbon flux through the acetyl-CoA node in an aerobic methanotroph, which opens

avenues for expanding the product range possible via bio-conversion of natural gas.

## 2. Materials and methods

### 2.1. Strains, plasmids, and genetic manipulation

Strain *Methylobacterium buryatense* 5GB1C, a variant of strain 5GB1 intentionally cured of its native plasmid (Puri et al., 2015), and *M. buryatense* 5GB1C  $\Delta$ pta (Gilman et al., 2017) were used as the host strains. *E. coli* MG1655 genes *atoB*, *fadB* (Clomburg et al., 2012) and *ydjI* (Kim et al., 2016), *Clostridium acetobutylicum* genes *hbd* and *crt* (Boynton et al., 1996), *Methanosaeta thermophila* gene *acs* (Berger et al., 2012), and codon optimized *NphT7* gene from *Streptomyces* sp. Okamura et al. (2010) synthesized by IDT DNA technologies, were cloned into the pAWP87 vector (Puri et al., 2015) with varying ribosome binding sites (RBS1, RBS2, RBS3, RBS4 and RBS5) upstream of the genes, and under the control of constitutive methanol dehydrogenase promoter (*P<sub>mxaf</sub>*), obtaining constructs pCA01–10. RBS1–5 were designed using RBS calculator v2.0 (Salis et al., 2009; Espah Borujeni et al., 2014) by selecting *Methylobacterium extorquens* AM1 as a proxy host organism due to unavailability of *M. buryatense* 5GB1 in the RBS calculator program. Construction of the plasmids was performed using Gibson Assembly (New England Biolabs Inc., Ipswich, MA) as described previously (Gibson et al., 2009). The resulting plasmids and strains are listed in Table S1, primers used for molecular biology are listed in Table S2, and RBS used are listed in Table S3.

Molecular kits for plasmid mini-preparation and gel extraction were purchased from QIAGEN (Hilden, Germany). DNA sequencing was outsourced to Lone Star Labs (Houston, TX). Transconjugation of host *M. buryatense* 5GB1C was performed with *Escherichia coli* S17-1  $\lambda$  pir acting as the donor strain as previously described (Puri et al., 2015).

### 2.2. Medium, culture and fermentation conditions

*M. buryatense* 5GB1C cells were routinely cultured in NMS2 medium (Puri et al., 2015) at 30 °C with orbital shaking at 250 rpm unless otherwise stated. 99.5% pure CH<sub>4</sub> (Airgas, Houston, TX, USA) was used as the sole carbon source for all cultures. Strains were grown in 250 ml glass serum bottles (Kimble Chase, Vineland, NJ, USA) with 25% (v/v) CH<sub>4</sub> in air sealed with rubber stoppers and aluminum seals (Wheaton, Millville, NJ, USA). Plates were incubated in sealed jars (Oxoid Limited, Hampshire, United Kingdom) in an atmosphere of 25% CH<sub>4</sub> (v/v) in air at 30 °C. Antibiotics (50  $\mu$ g/ml of kanamycin or 30  $\mu$ g/ml rifampicin) were added to the growth medium as required.

The seed culture was prepared by inoculating a single colony from a freshly grown plate in 30 ml NMS2 medium with 25% (v/v) CH<sub>4</sub> in air and incubated for 24 h. The primary pre-cultures were inoculated from this seed culture at 1% (v/v) into 250 ml serum bottle containing 40 ml NMS2 medium and grown with 25% (v/v) CH<sub>4</sub> in air. After 28 h, cells were harvested by centrifugation (10 min at 5875 g) and re-suspended in 20 ml fresh NMS2 medium to an OD of 30. During cultivation and fermentation, the gas phase of the serum bottle was refreshed every 24 h by opening the bottles, re-sealing, removing 40 ml of air and injecting 60 ml of methane through the rubber seal septum. This resulted in a headspace containing 25% methane (v/v), 16% oxygen, and balance nitrogen. Fermentations were monitored for 120 h via culture samples collected every 24 h.

For product toxicity experiments, crotonic, butyric and acetic acids (Sigma-Aldrich, St. Louis, MO) at concentrations 0.1, 0.2, 0.5 and 1 g/L were added to *M. buryatense* 5GB1C cultures grown in 20 ml NMS2 medium in glass serum bottles starting at an initial OD 0.05, incubated at 30 °C at 200 rpm in an orbital shaker.

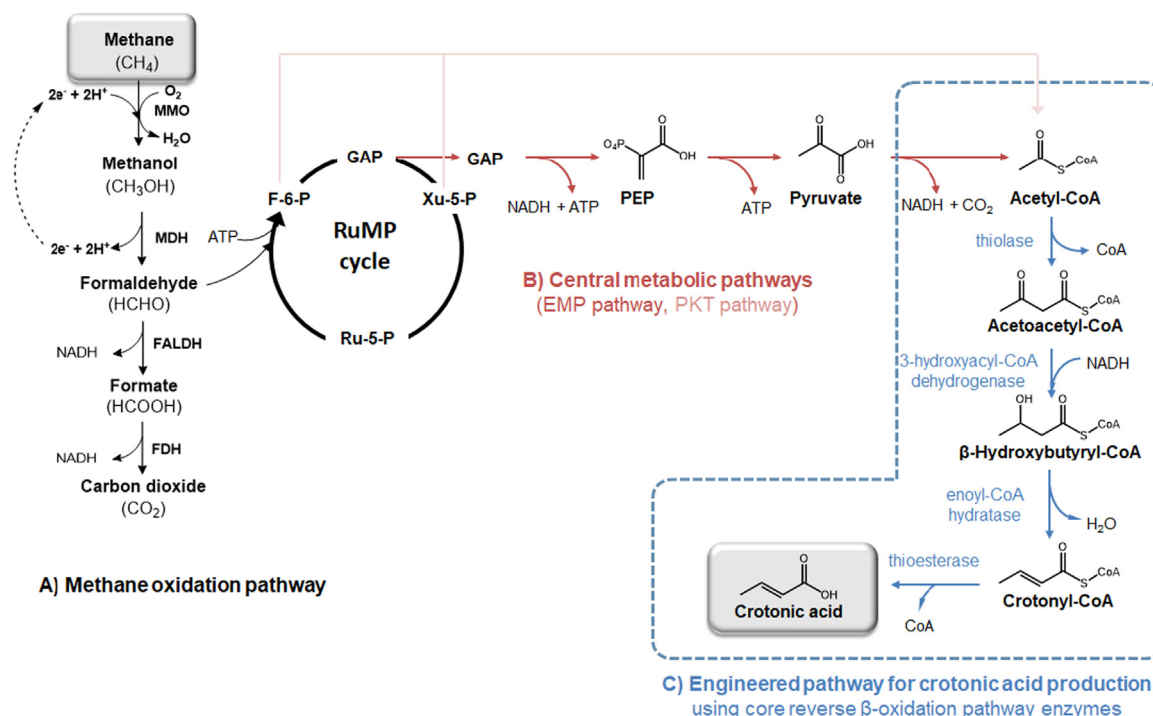


**Fig. 1.** Product synthesis from acetyl-CoA node using engineered reverse  $\beta$ -oxidation pathway in *M. buryatense* 5GB1C. Methane oxidation pathway and central metabolic pathways consisting of Ribulose Monophosphate cycle pathway (RuMP; bold, black arrows), Embden-Meyerhoff-Parnas pathway (EMP; red arrows) and Phosphoketolase pathway (PKT; pink arrows) that are native to *M. buryatense* 5GB1C are shown (A, B). Engineered pathway for crotonic acid production from acetyl-CoA node is shown in blue (C). Abbreviations: MMO: Methane monooxygenase, MDH: Methanol Dehydrogenase, FALDH: Formaldehyde Dehydrogenase, FDH: Formate Dehydrogenase, Ru-5-P: Ribulose-5-Phosphate, Xu-5-P: Xylulose-5-Phosphate, F-6-P: Fructose-6-Phosphate, GAP: Glyceraldehyde-3-Phosphate, PEP: Phosphoenolpyruvate.

### 2.3. Analytical methods

Cell density was estimated by measurement of the optical density of appropriately diluted culture samples at 600 nm using a Beckman Coulter DU 800 UV/Vis Spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). The OD to cell dry weight correlation was determined to be 0.24 gCDW/OD/L.

The concentrations of extracellular metabolites (acetate, formate, crotonate and butyrate) in the culture supernatant were determined via HPLC as previously described (Clomburg et al., 2012). Briefly, HPLC was conducted using a Shimadzu Prominence SIL 20 system (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) equipped with an HPX-87H organic acid column (Bio-Rad, Hercules, CA, USA) with operating conditions to optimize peak separation. A mobile phase of 30 mM  $\text{H}_2\text{SO}_4$  at a 0.3 ml/min flow rate was used and the column was operated at 42 °C.

For GC-FID and GC-MS confirmation of crotonic and butyric acid, transesterification and derivatization of culture samples to fatty acid methyl esters (FAME) were performed as described previously (Kim et al., 2016). GC analysis was conducted on an Agilent 7890B Series Custom Gas Chromatography system equipped with a 5977B Inert Plus Mass Selective Detector Turbo EI Bundle (for identification) or a Flame Ionization Detector (for quantification) and an Agilent HP-5-MS capillary column (0.25 mm internal diameter, 0.25  $\mu\text{m}$  film thickness, 30 m length). The following temperature profile was used with helium as the carrier gas at a flowrate of 1.5 ml/min: Initial 50 °C (hold 3 min); ramp at 20 °C/min to 270 °C (hold 6 min). The injector and detector temperatures were 250 °C and 350 °C, respectively. 1  $\mu\text{l}$  of sample was injected with a 4:1 split ratio.

### 2.4. Enzyme assays

For enzyme assays, *M. buryatense* 5GB1C cells were grown to mid-

exponential phase, pelleted and re-suspended to OD 40 in 100 mM Tris-HCl buffer (pH 7.0) with 1 mM Dithiothreitol (DTT). Re-suspended cells were lysed by adding 0.75 g glass beads to 1 ml of cell suspension in 1.5 ml centrifuge tube followed by homogenization of the mixture in a Disruptor Genie (Scientific Industries, Inc, Bohemia, NY), and centrifugation at 15,000  $\times g$  at 4 °C for 10 min to pellet cell debris. The supernatant was used as the cell extract for measurement of enzyme activities. Enzyme assays for thiolase, 3-hydroxybutyryl-CoA dehydrogenase, crotonase and thioesterase activities were performed as described previously (Clomburg et al., 2012; Vick et al., 2015). Briefly, thiolase (AtoB, EC 2.3.1.9) activity was measured by monitoring the decrease in acetoacetyl-CoA at 303 nm. 3-hydroxybutyryl-CoA dehydrogenase (FadB/Hbd, EC 1.1.1.35) activity was measured by monitoring the decrease in NADH at 340 nm using acetoacetyl-CoA as the substrate. Crotonase (Crt, EC 4.2.1.17) activity was measured by monitoring crotonyl-CoA absorbance at 263 nm (Hartmanis and Gatenbeck, 1984). Thioesterase (YdiI, EC 3.1.2.22) activity was measured by monitoring reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) at 412 nm using crotonyl-CoA as the substrate. All assays were carried out in 200  $\mu\text{l}$  reaction volume in 96 well micro-titer plates at 30 °C using Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT). Protein concentration in cell extracts was determined using Bradford reagent (BioRad, Hercules, CA).

## 3. Results

### 3.1. Engineering *M. buryatense* 5GB1C for crotonic acid production

*M. buryatense*, an obligate methanotroph, assimilates carbon from methane via the ribulose monophosphate pathway (RuMP) that combines formaldehyde with ribulose-5-phosphate resulting in the formation of fructose-6-phosphate. This hexose-phosphate further enters the 'sugar' linked central metabolic pathways of *M. buryatense* resulting in

central metabolites such as phosphoenolpyruvate, pyruvate and acetyl-CoA, each of which can serve as a node for production of bio-based chemicals and fuels. Additionally, the recently discovered phosphoketolase pathway (Henard et al., 2015) also contributes to acetyl-CoA formation from sugar-phosphates of the RuMP pathway (Fig. 1). To demonstrate production of an industrial platform chemical from the acetyl-CoA node in *M. buryatense* 5GB1C, we sought to engineer a reverse  $\beta$ -oxidation based metabolic pathway for crotonic acid production (Fig. 1). This pathway has been demonstrated in the industrial strain *Escherichia coli* (Kim et al., 2016; Clomburg et al., 2012) wherein a thiolase (AtoB) initiates the pathway by condensing two molecules of acetyl-CoA to generate acetoacetyl-CoA, followed by a 3-hydroxyacyl-CoA dehydrogenase (FadB) that reduces acetoacetyl-CoA to 3-hydroxybutyryl-CoA, which is dehydrated by an enoyl-CoA hydratase (FadB) to generate crotonyl-CoA. Finally, an enoyl-CoA specific thioesterase (YdiI) cleaves the thiol group of crotonyl-CoA resulting in the free unsaturated fatty acid crotonic acid (Fig. 1).

For engineering this pathway, *atoB*, *fadB* and *ydiI* genes from *E. coli* MG1655 were initially selected to catalyze the thiolase, 3-hydroxyacyl dehydrogenase/enoyl-CoA hydratase and thioesterase reactions, respectively, as these genes have previously been used in *E. coli* for successful demonstration of crotonic acid production from acetyl-CoA (Kim et al., 2016). To optimize the expression level of each enzyme in the heterologous pathway, we used RBS variants of different strengths designed using RBS calculator 2.0 program (Salis et al., 2009; Espah Borujeni et al., 2014). This approach of varying RBS to optimize translation initiation rate and thereby increasing final product titers has been used previously for chemical production in *E. coli* (Nowroozi et al., 2014). Additionally, we also varied the order of genes as gene clusters may have a bearing on transcription and may interfere in translation, resulting in altered levels of final product (Smanski et al., 2014). Thus, by using a different ribosome binding site for each gene, and varying the order of the three genes on the pAWP87 plasmid (Puri et al., 2015) under the control of a constitutive methanol dehydrogenase promoter (*P<sub>mxsF</sub>*) (Puri et al., 2015), four strains were obtained, namely 5GB1C (pCA01), 5GB1C (pCA02), 5GB1C (pCA03) and 5GB1C (pCA04)

(Fig. 2A).

Functional expression of AtoB, FadB and YdiI in *M. buryatense* 5GB1C was confirmed via enzyme activity assays that showed increased thiolase ( $\sim 20$   $\mu\text{mol}/\text{mg}/\text{min}$ ) and thioesterase ( $\sim 5$ – $6$   $\text{nmol}/\text{mg}/\text{min}$ ) activities compared to the control (Fig. 2B). FadB activity (as measured by  $\beta$ -ketoacyl-CoA reduction) was detected at lower levels (0.05–0.1  $\mu\text{mol}/\text{mg}/\text{min}$ ) than those observed previously in *E. coli* (Vick et al., 2015) (Fig. 2B). Differences in the specific activities measured for each protein in response to the gene configuration and RBS utilized were observed, with minimal variation in thiolase and thioesterase activities (less than 25%) while FadB activity showed a near 2-fold variation depending on the configuration and RBS combination (Fig. 2B). Despite overall low FadB activity, crotonate production was detected via HPLC, and confirmed by GC-MS (Supplementary Fig. S1), in all four strains at titers ranging between 60 and 70 mg/L. No crotonic acid production was observed in the control strain containing the empty vector. Normalizing the crotonic acid titer relative to dry cell weight, 90–190  $\mu\text{mol}$  crotonate/gCDW was produced by these strains (Fig. 2C). Interestingly, the time at which crotonic acid first appeared varied with each strain. For example, 5GB1C (pCA01) showed crotonic acid production only after 120 h, whereas variations in RBS and gene order afforded faster production, with 5GB1C (pCA03) and 5GB1C (pCA04) forming crotonic acid as early as 48 h (Fig. 2C). Notably, the strains containing the 5GB1C (pCA03) and 5GB1C (pCA04) constructs had nearly 2-fold higher FadB activity compared to 5GB1C (pCA01) (Fig. 2B). During the course of cultivation, the OD remained stable, and pH decreased to 8.0–8.5 from an initial pH of 9.5.

In addition to crotonic acid, expected by-products such as acetate and formate and the unexpected by-product butyrate were detected in culture medium of these strains. All four strains primarily formed acetate (0.05–0.2 g/L) and formate (0.01–0.02 g/L) prior to producing crotonic acid. Butyric acid at titers of approximately 0.04 g/L was detected either at the time of crotonic acid production (in 5GB1C (pCA04)) or after formation of crotonic acid (in 5GB1C (pCA01), 5GB1C (pCA02) and 5GB1C (pCA03)). The identity of butyric acid was confirmed via GC-MS (Supplementary Fig. S2). The empty vector

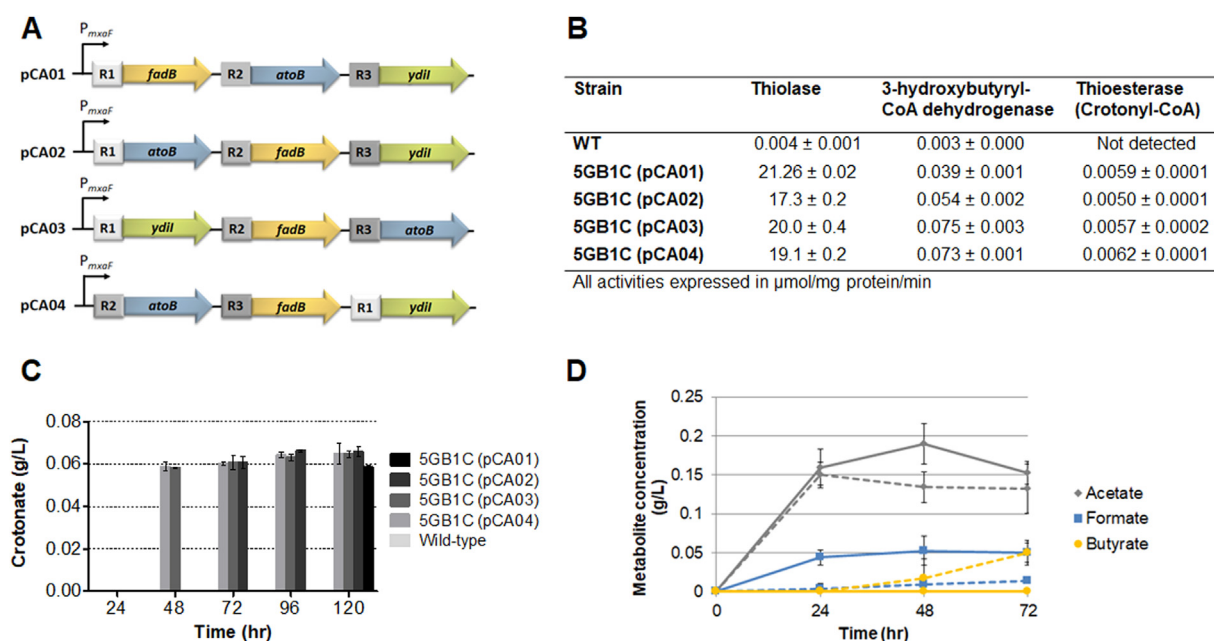


**Fig. 2.** Production of crotonic acid in engineered *M. buryatense* 5GB1C strains. Four strains expressing *atoB*, *fadB* and *ydiI* genes with varying RBS and gene-orders under the control of constitutive *mxsF* promoter (*P<sub>mxsF</sub>*) were designed (A). Expression of thiolase (AtoB), 3-hydroxybutyryl-CoA dehydrogenase (FadB), thioesterase (YdiI) in *M. buryatense* 5GB1C was confirmed via enzyme activity assays (B). Crotonate production profile is shown for all four strains grown in 20 ml culture medium in 250 ml glass serum bottles at high cell density (OD 30), 30 °C, 200 rpm with 25% methane-in-air with headspace refreshed every 24 h (C) Additional by-products profiles of the best performing strain, 5GB1C (pCA04), (dashed lines) is compared with that of the empty vector control strain (solid lines) (D). Each data point is average of replicate experiments, and error bars represent standard deviation.

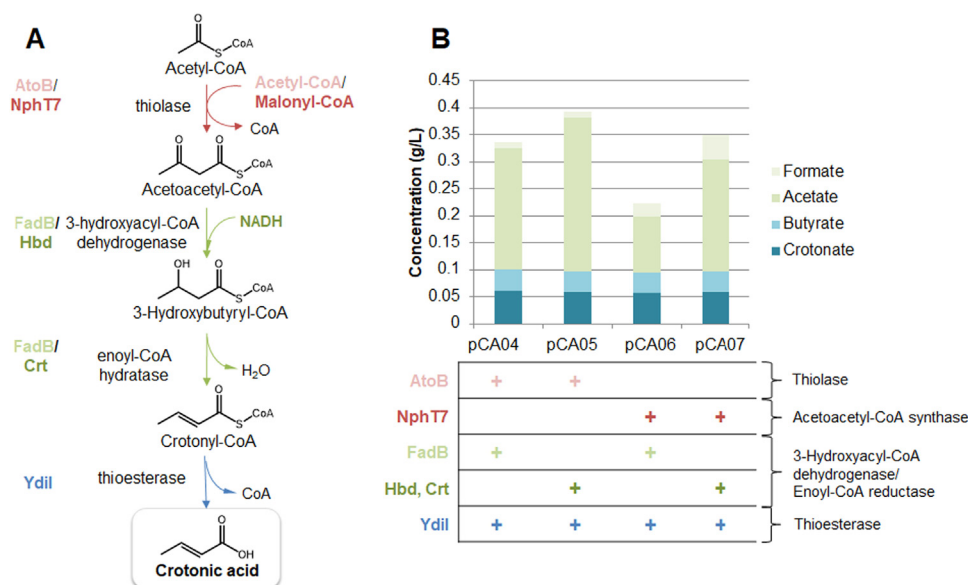


**Fig. 3.** Expression of alternative genes for crotonic acid production in *M. buryatense* 5GB1C via acetyl-CoA node. Genetic manipulations are shown that target replacement of *E. coli* MG1655 *atoB* with *Streptomyces sp. NphT7* for acetoacetyl-CoA synthesis, *E. coli* MG1655 *fadB* with *hbd*, *crt* from *Clostridium acetobutylicum* for 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase reactions in engineered *M. buryatense* 5GB1C host (A). Evaluation of metabolite production profiles (at 72 h) in resultant strains harboring plasmids pCA05, pCA06 and pCA07, as compared to strain with parent plasmid, pCA04 (B).

control strain formed acetate and formate but did not accumulate butyrate or crotonate (Fig. 2D). This suggests that the engineered strains accumulated butyric acid most likely by action of an endogenous enoyl-CoA reductase that reduced crotonyl-CoA to butyryl-CoA that subsequently underwent thiol cleavage reaction catalyzed either by a native thioesterase, or the recombinant thioesterase, YdiI which has been shown to be highly specific for four carbon-chain acyl-CoAs (Kim et al., 2016).

As strain 5GB1C (pCA04) formed both crotonic acid and butyric acid at 48 h, the earliest timepoint at which crotonic acid was observed, this strain was selected for further genetic manipulations aimed at increasing crotonic acid titers.

### 3.2. Alternative genes to enhance crotonic acid production

To improve crotonic acid titers, several gene replacements were attempted targeting replacement of *E. coli* *atoB* and *fadB* genes with alternative versions from other organisms (Fig. 3A). Specifically, as low FadB activity was detected in the initial strains 5GB1C (pCA01–04), we first sought to replace *E. coli* MG1655 *fadB* with NADH-dependent Hbd and Crt from *Clostridium acetobutylicum* that are involved in butyryl-CoA synthesis from acetyl-CoA (Boynton et al., 1996; Hartmanis and Gatenbeck, 1984), generating strain 5GB1C (pCA05). These enzymes have been utilized in numerous metabolic engineering studies aimed at C4 product synthesis (e.g. butanol) due to their high specificity for four-carbon intermediates (Shen et al., 2011). Additionally, given the thermodynamic constraints of the condensation reaction (Lan and Liao, 2012) and the potential for the acetyl-CoA pool to be a limiting factor, we replaced *E. coli* AtoB that catalyzes reversible condensation of two molecules of acetyl-CoA, with a KASIII homolog, the acetoacetyl-CoA synthase (EC 2.3.1.194) NphT7 from soil bacterium *Streptomyces sp. CL190*, that carries out the ATP-dependent irreversible condensation of malonyl-CoA with acetyl-CoA to form acetoacetyl-CoA (Okamura et al., 2010). As *M. buryatense* 5GB1 forms relatively high amounts of lipids (8% FAME content per g CDW) compared to many other bacteria (Demidenko et al., 2016), we reasoned that this organism may have a large pool of malonyl-CoA available for NphT7 driven condensation with acetyl-CoA. Strain 5GB1C (pCA06) was constructed that expressed NphT7 in place of AtoB, and NphT7 was further combined with Hbd, Crt to generate strain 5GB1C (pCA07).

However, these gene replacements did not improve crotonic or butyric acid titers compared to the parent strain, 5GB1C (pCA04) (Fig. 3B). As FadB activity was low in the parent strain, we determined

3-hydroxybutyryl-CoA dehydrogenase activity levels in all the strains expressing alternative versions of FadB, i.e. Hbd and Crt. Enzyme activity assays of 3-hydroxybutyryl-CoA dehydrogenase in 5GB1C (pCA05) and 5GB1C (pCA07) expressing Hbd and Crt revealed slightly higher activity,  $0.150 \pm 0.048$   $\mu\text{mol}/\text{min}/\text{mg}$  and  $0.133 \pm 0.029$   $\mu\text{mol}/\text{min}/\text{mg}$  in the two strains, respectively, compared to  $0.073 \pm 0.001$   $\mu\text{mol}/\text{min}/\text{mg}$  in the parent strain, 5GB1C (pCA04). However, crotonase activity in both 5GB1C (pCA05) and 5GB1C (pCA07) was low,  $0.007 \pm 0.004$   $\mu\text{mol}/\text{min}/\text{mg}$  and  $0.027 \pm 0.002$   $\mu\text{mol}/\text{min}/\text{mg}$  respectively, which could be responsible for low flux through the pathway. Even though crotonic and butyric acid levels were unaffected, differences were noted in the number of by-products produced by these strains (Fig. 3B). Strain 5GB1C (pCA05) expressing *atoB*, *hbd*, *crt* and *ydiI* formed the highest amount of acetate compared to other strains that expressed *nphT7* with *fadB/hbd,crt* and *ydiI*. Specifically, 5GB1C (pCA05) formed 0.3 g/L acetate (equivalent to  $\sim 500$   $\mu\text{mol}/\text{gCDW}$ ) which was 3-fold and 1.5-fold higher than that produced by 5GB1C (pCA06) and 5GB1C (pCA07) respectively. The amount of formate generated by 5GB1C (pCA05) (0.01 g/L) was also lower than that formed by 5GB1C (pCA06) (0.02 g/L) and 5GB1C (pCA07) (0.04 g/L). Given that 5GB1C (pCA05) channeled more carbon to acetate formation and with lower formate production compared to other strains, this strain was chosen for further genetic manipulations targeted at blocking acetate formation and up-regulating acetate conversion to acetyl-CoA, thus potentially improving carbon flux through acetyl-CoA node.

### 3.3. Genetic manipulations to alter acetate formation and acetate uptake

To increase crotonate production, the excess carbon being lost to acetate could be recovered and channeled towards crotonate either by i) blocking the pathway for acetate formation by deleting the phosphotransacetylase (*pta*) gene in the host strain, a strategy commonly used to reduce the flux to acetate (Dittrich et al., 2005; Shams Yazdani and Gonzalez, 2008; Singh et al., 2011), or ii) by recycling acetate back to acetyl-CoA by overexpressing acetyl-CoA synthetase (*acs*). The latter strategy has been reported in literature as a tool for lowering acetate accumulation in *E. coli* and in *Saccharomyces cerevisiae* (Lin et al., 2006; Shiba et al., 2007). Both of these strategies were evaluated to improve crotonic acid production in *M. buryatense* 5GB1C (Fig. 4A).

The plasmid expressing *atoB*, *hbd*, *crt* and *ydiI* genes (pCA05) was introduced into an engineered *M. buryatense* 5GB1C host strain containing a *pta* deletion and product formation was evaluated. While

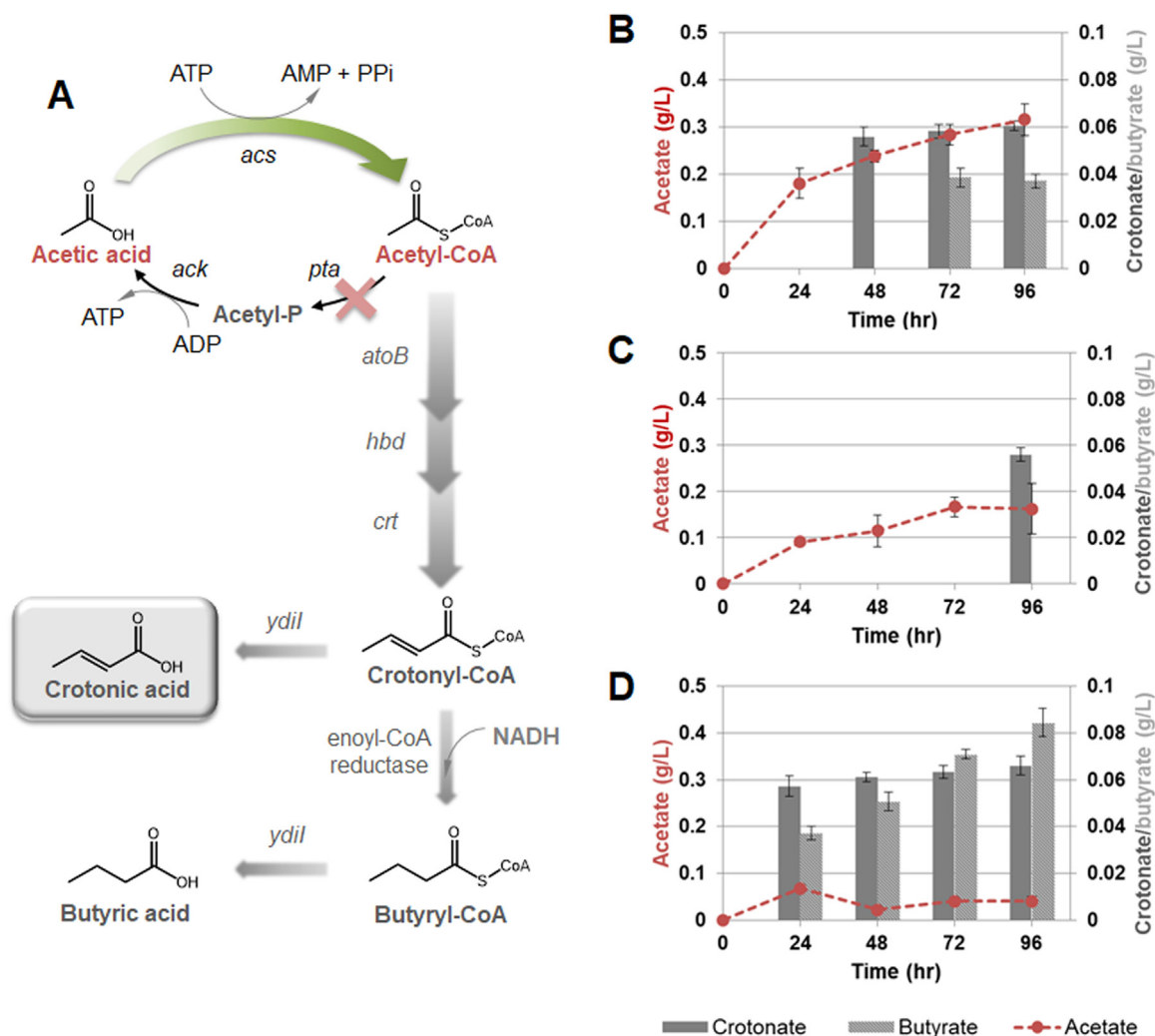


**Fig. 4.** Genetic manipulation of acetate formation and uptake system to increase crotonic acid production in engineered *M. buryatense* 5GB1C. Pathway showing genetic manipulations incorporated in *M. buryatense* 5GB1C host strain to i) block acetate formation by deleting *pta* gene, or ii) re-utilize acetate by over-expressing *acs* from *E. coli* or *Methanosaeta thermophila* (A). Metabolite profile of 5GB1C (pCA05) is shown (B), impact of  $\Delta pta$  gene deletion on acetate formation and 4-C carboxylic acids formation in 5GB1C strain expressing pCA05 plasmid (C), and impact of *M. thermophila acs* over-expression in combination with *atoB*, *hbd*, *crt* and *ydiI* in 5GB1C (pCA09) is shown (D). Each data point is average of replicate experiments, and error bars represent standard deviation.

acetate production was reduced approximately 50% in the  $\Delta pta$  host strain harboring pCA05 compared to the wild-type host strain harboring the same plasmid, there was no improvement in crotonic acid titer (Figs. 4B, 4C). Furthermore, crotonic and butyric acid production was not observed until 96 h and 120 h, respectively.

In an alternative strategy aimed at assimilating acetate, an AMP-forming acetyl-CoA synthetase gene sourced either from *E. coli* (Kumari et al., 1995) or *Methanosaeta thermophila*, a thermophilic methanogen, (Berger et al., 2012) was overexpressed in combination with *atoB*, *hbd*, *crt* and *ydiI* genes resulting in strains 5GB1C (pCA08) and 5GB1C (pCA09), respectively. While strain 5GB1C (pCA08) that over-expressed *E. coli acs* did not show any crotonic or butyric acid production, strain 5GB1C (pCA09) over-expressing *M. thermophila acs* formed crotonic and butyric acids as early as 24 h. Even though crotonic acid titer in 5GB1C (pCA09) was similar to that produced by the parent strain, 5GB1C (pCA05), butyric acid titer increased to 0.08 g/L by 96 h in 5GB1C (pCA09), a 2-fold increase compared to 5GB1C (pCA05) (Figs. 4B, 4D). Acetate production in 5GB1C (pCA09) was reduced by 60–90% compared to 5GB1C (pCA05) (Figs. 4B, 4D). A cumulative titer of 0.15 g/L of 4-C carboxylic acids was eventually obtained in 5GB1C (pCA09) strain after 96 h through overexpression of an acetyl-CoA synthetase aimed at shuttling carbon from acetate back to acetyl-CoA.

#### 3.4. Process optimization to improve crotonic acid production

Using the 5GB1C (pCA09) strain, various process modifications were evaluated for their impact on product formation. Changing the methane-to-air ratio in the headspace, increasing nitrate concentration from  $1 \times$  to  $8 \times$  in the medium (to overcome nitrogen limitation if any), and variation in culture volume (10, 15, 30 ml) did not improve crotonic acid production (data not shown).

Product toxicity was also evaluated by growing wild-type *M. buryatense* 5GB1C in varying concentrations of acetic, butyric and crotonic acids. While acetic and butyric acids up to 0.5 g/L did not impact *M. buryatense* 5GB1C's growth, 1 g/L of both these acids resulted in slight growth impairment compared to the control to which no acids were added (Fig. 5A, B). In contrast, crotonic acid above 0.1 g/L proved to be highly inhibitory to *M. buryatense*'s growth (Fig. 5C). While the maximum amount of crotonic acid formed in this study is less than 0.1 g/L, suggesting that bottlenecks in the pathway, rather than product toxicity, prevent accumulation/formation of crotonic acid at titers higher than 0.07 g/L, this toxicity is an important consideration in further improving crotonic acid production. In addition to improving pathway function (see Section 4), strain engineering efforts can possibly target alteration of membrane fluidity to block toxic product entry into

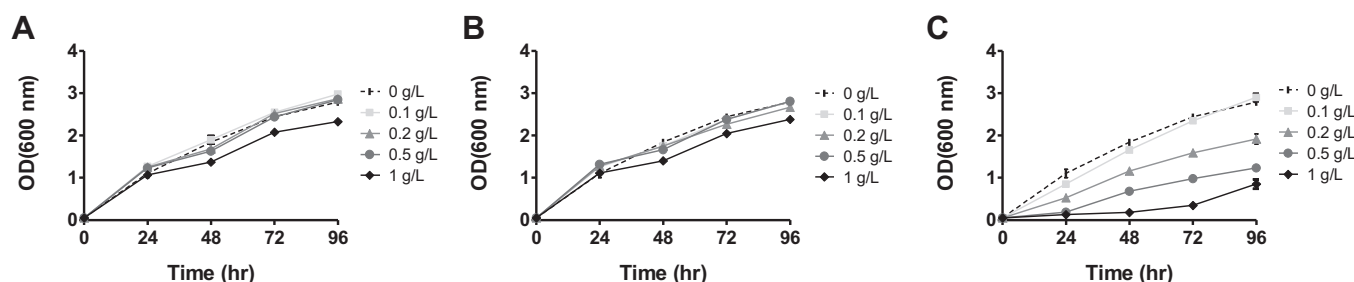


Fig. 5. Crotonic, butyric and acetic acids tolerance of *M. buryatense* 5GB1C. Impact of varying concentrations (0, 0.1, 0.2, 0.5 and 1 g/L) of acetic acid (A), butyric acid (B) and crotonic acid (C) on growth of *M. buryatense* 5GB1 is shown. Each data point is average of two replicates, and error bars represent standard error.

cells, and heterologous expression of effluent pumps to remove excess product out of cells (Dunlop, 2011). Furthermore, a rational approach aimed at identifying toxicity targets of crotonic acid in combination with random mutagenesis to identify crotonic acid tolerant mutants can also be used (Connor et al., 2010; Stephanopoulos, 2002). Adaptive lab evolution can also be used to improve strain tolerance to increased crotonic acid concentrations (Hu et al., 2016). Understanding the mechanism(s) of product toxicity and these types of approaches to alleviate toxic effects will be critical for both crotonic acid production as well as other non-native products that can potentially be produced via metabolic engineering of this methanotroph.

#### 4. Discussion

In this study, we have demonstrated product formation from the acetyl-CoA node through heterologous pathway expression in an obligate methanotroph using methane as the carbon source. For this, a modified version of the reverse  $\beta$ -oxidation (r-BOX) pathway (Dellomonaco et al., 2011) was used where one turn of the r-BOX cycle was combined with an enoyl-CoA specific cycle termination thioesterase, YdiI, (Kim et al., 2016) to generate the four carbon  $\alpha,\beta$ -unsaturated carboxylic acid crotonic acid from crotonyl-CoA. In addition to crotonic acid, the four-carbon saturated carboxylic acid butyric acid was also formed as the by-product of the engineered pathway, with the cumulative titer of these two products reaching 0.15 g/L in the best performing strain. The formation of butyrate from crotonic acid was unexpected as no *trans*-enoyl-CoA reductase (TER) was over-expressed. This suggests that reduction of crotonyl-CoA to butyryl-CoA is being driven by an endogenous *trans*-enoyl-CoA reductase. Previously, it has been shown that *E. coli* enoyl-ACP reductase (FabI) possesses inherent TER activity (Vick et al., 2015) to support reduction of crotonyl-CoA to butyryl-CoA. It is possible that *M. buryatense* FabI (METBU\_RS0100175, NCBI Reference Sequence: NZ\_KB455575.1) also possesses TER activity similar to that possessed by *E. coli* FabI, thus catalyzing reduction of crotonyl-CoA to butyryl-CoA, thereby leading to butyrate production, most likely by the action of over-expressed YdiI on butyryl-CoA.

Despite the successful demonstration of product synthesis from the acetyl-CoA node, the overall titers achieved remain low. Based on the measurement of specific activities of each enzyme involved in the pathway from acetyl-CoA to crotonic acid, our results indicate that functional expression levels of heterologous genes may limit pathway performance in this organism. Specifically, the measured 3-hydroxybutyryl-CoA dehydrogenase activities, regardless of the enzyme utilized (i.e. FadB or Hbd), were significantly lower (by more than an order of magnitude) than those observed upon overexpression of these enzymes in *E. coli* (Vick et al., 2015; Shen et al., 2011). In contrast, thiolase activity observed upon AtoB overexpression in *M. buryatense* is remarkably similar to that obtained in *E. coli* (Vick et al., 2015; Shen et al., 2011), indicating that functional expression of enzymes in this methanotroph may be highly enzyme specific. Thus, future approaches to improve strain performance can target evaluation of additional gene variants for each step of the pathway including evaluation of codon

usage in these genes to determine the best gene candidates. Our results also showed that varying RBS and gene order impacted the rate of product formation. This means while a complete understanding of *M. buryatense* physiology is still lacking, a combinatorial gene assembly approach as used in *E. coli* and *S. cerevisiae*, by varying promoters, RBS, gene order and gene variants, for heterologous expression of multiple genes (Nowroozi et al., 2014; Zhang et al., 2015; Keasling, 2012) would be useful for future metabolic engineering efforts in *M. buryatense* 5GB1. These efforts can be aided by a more rigorous examination of transcriptional and translational responsiveness of gene and protein expression to various synthetic designs (e.g. promoters, RBS, gene configuration, etc.) to provide an improved understanding of key elements for optimal heterologous gene expression.

In addition to functional expression of the desired pathway enzymes, the availability of acetyl-CoA also appears to be a major factor dictating product synthesis. Our experiments showed that while deleting phosphotransacetylase reduced acetate formation, crotonic and butyric acid formation were also reduced as a result. These results indicate that decreasing acetate formation through this approach did not enable increased acetyl-CoA availability. This supports the role and importance of phosphotransacetylase within the phosphoketolase pathway for acetyl-CoA formation in which phosphotransacetylase catalyzes the conversion of acetyl-phosphate to acetyl-CoA (Henard et al., 2015). Given the recent report demonstrating a 2-fold increase to the intracellular acetyl-CoA concentration and enhanced biomass and lipid accumulation via overexpression of a phosphoketolase isoform in *M. buryatense* (Henard et al., 2017), this pathway appears to play a large role in acetyl-CoA formation. As such, an alternative approach for reducing acetate formation while still retaining phosphotransacetylase for converting acetyl-phosphate to acetyl-CoA can be explored through the deletion of acetate kinase.

In contrast to a *pta* deletion, over-expression of *M. thermophila acs* (Berger et al., 2012) for the conversion of acetate to acetyl-CoA enabled increased C4-carboxylic acid formation, demonstrating the importance of increasing acetyl-CoA availability and viability of this approach for this purpose. To further increase flux through acetyl-CoA node, additional pathways that divert carbon away from acetyl-CoA can be blocked (Kalyuzhnaya et al., 2015). For example, by deleting lactate dehydrogenase, more pyruvate can be channeled to acetyl-CoA formation via EMP pathway. Additionally, elimination of the glycogen synthesis pathway can also increase availability of carbon for central metabolites formation. *M. buryatense* also has a highly active fatty acid synthesis pathway that draws from the acetyl-CoA pool (Demidenko et al., 2016), thus down-regulation of the FAS pathway, in combination with deletion of native beta-oxidation pathway genes (Demidenko et al., 2016) could further improve acetyl-CoA available for target product synthesis. However, the alteration of FAS pathway flux may have the unintended consequence of limiting intracellular membrane formation thereby inhibiting particulate methane monooxygenase's functioning. In such a case, strategies such as switching to copper-free cultivation of *M. buryatense* to activate sMMO expression, or fine-tuning of FAS down-regulation to enable sufficient expression of pMMO can be

explored.

Increased knowledge on the physiology and metabolic network of *M. buryatense* can also aid in optimizing flux to maximize acetyl-CoA and target product synthesis. Based on experimental data, two routes are known to generate acetyl-CoA in *M. buryatense*: i) EMP pathway where pyruvate dehydrogenase converts pyruvate to acetyl-CoA (Kalyuzhnaya et al., 2013), and ii) PKT pathway where FPkt or XPkt catalyze conversion of fructose-6-phosphate or xylulose-5-phosphate to acetyl-CoA (Henard et al., 2015). Each of these pathways have varying requirements for reducing equivalents, and the current lack of knowledge about the exact requirements of reducing equivalents for ATP generation in *M. buryatense* 5 GB1 limits metabolic engineering efforts for optimizing flux through these three pathways. Theoretically, by modulating the carbon flux through these pathways, different carbon and energy efficiencies can be achieved. For example, if acetyl-CoA is primarily formed through the EMP pathway, then production of 1 mol of crotonyl-CoA results in the net generation of 3 NADH molecules, 2 ATP and 2 CO<sub>2</sub> per 6 mol of formaldehyde entering the RuMP pathway, achieving 67% carbon efficiency. At this maximum theoretical carbon efficiency, for every mole of crotonic acid produced, 6 mol of methane are consumed and 2 mol of CO<sub>2</sub> released. In contrast, the formation of crotonyl-CoA from the PKT pathway consumes 1 NADH per 4 mol of formaldehyde, with 100% carbon conversion efficiency (4HCHO + NADH → Crotonyl-CoA). By distributing carbon flux through these two pathways (equations (i), (ii) and (iii)), a redox balanced and ATP generating conversion of formaldehyde to crotonyl-CoA can be achieved with maximum theoretical carbon efficiency of 89%.

3HCHO → 0.5Crotonyl-CoA + CO<sub>2</sub> + ATP + 1.5NADH (i) EMP pathway

6HCHO + 1.5NADH → 1.5Crotonyl-CoA (ii) PKT pathway

9HCHO → 2Crotonyl-CoA + CO<sub>2</sub> + ATP (iii) = (i) + (ii)

Determination of flux distribution between these pathways and understanding of reducing equivalent requirements by *M. buryatense* 5 GB1 can enable future efforts targeting improved carbon efficiency and restoration of redox balance through these pathways, in combination with regulating the expression of formate dehydrogenase and formaldehyde dehydrogenase genes.

Furthermore, based on genome scale metabolic reconstruction of *M. buryatense* a partial serine cycle is predicted to contribute to the acetyl-CoA pool (de la Torre et al., 2015), indicating overexpression of serine cycle enzymes may also improve titers of products formed through acetyl-CoA. Per mole of formaldehyde, the serine cycle consumes a mole of CO<sub>2</sub> to produce a mole of acetyl-CoA while consuming NADH and ATP (Kalyuzhnaya et al., 2015) (equation iv). Thus, while activation of the serine pathway for crotonic acid production may allow capturing of CO<sub>2</sub> along with CH<sub>4</sub>, this pathway will be very demanding in terms of reducing equivalents and ATP requirements (equation vi) making it unfavorable for target product synthesis.

HCHO + CO<sub>2</sub> + 2NADPH + 3ATP → Acetyl-CoA (iv) Serine cycle  
2Acetyl-CoA + NADH → Crotonyl-CoA (v) Crotonic acid production pathway

2HCHO + 2CO<sub>2</sub> + 4NAD(P)H + NADH + 6ATP → Crotonyl-CoA (vi) = (iv) + (v)

Building on the results of this study, which has demonstrated product synthesis from acetyl-CoA node in an obligate methanotroph, these types of approaches can be exploited to both improve product synthesis and open up avenues for further diversifying target products from acetyl-CoA node (Kalyuzhnaya et al., 2015). For example, by utilizing multiple turns of r-BOX cycle, medium chain saturated and unsaturated carboxylic acids can be produced (Kim et al., 2015), and by varying starter substrates for the r-BOX pathway or for fatty acid synthesis pathway, functionalized carboxylic acids and alcohols can be generated

(Cheong et al., 2016; Garg et al., 2016). Implementation of these approaches for improving product synthesis and diversification will also rely on further advancement in the understanding of and ability to engineer *M. buryatense* to unlock the potential of this organism for the biological conversion of methane to fuels and chemicals.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ymben.2018.06.001>.

## References

- Anthony, C., 1982. The Biochemistry of Methylotrophs. Academic Press, London, New York, pp. 431.
- Berger, S., Welte, C., Deppenmeier, U., 2012. Acetate activation in *Methanosaeta thermophila*: characterization of the key enzymes pyrophosphatase and acetyl-coa synthetase. *Archaea* 2012, 315153.
- Blumenstein, J., Albert, J., Shulz, R.P., Kohlpaintner, C., 2015. Crotonaldehyde and Crotonic Acid. *Ullmann's Encyclopedia of Industrial Chemistry*.
- Bothe, H., et al., 2002. Heterotrophic bacteria growing in association with *Methylococcus capsulatus* (Bath) in a single cell protein production process. *Appl. Microbiol. Biotechnol.* 59, 33–39.
- Boynton, Z.L., Bennett, G.N., Rudolph, F.B., 1996. Cloning, sequencing, and expression of clustered genes encoding beta-hydroxybutyryl-coenzyme A (CoA) dehydrogenase, crotonase, and butyryl-CoA dehydrogenase from *Clostridium acetobutylicum* ATCC 824. *J. Bacteriol.* 178, 3015–3024.
- Cheong, S., Clomburg, J.M., Gonzalez, R., 2016. Energy- and carbon-efficient synthesis of functionalized small molecules in bacteria using non-decarboxylative Claisen condensation reactions. *Nat. Biotechnol.* 34, 556–561.
- Clomburg, J.M., Vick, J.E., Blankschien, M.D., Rodríguez-Moyá, M., Gonzalez, R., 2012. A synthetic biology approach to engineer a functional reversal of the β-oxidation cycle. *ACS Synth. Biol.* 1, 541–554.
- Clomburg, J.M., Crumley, A.M., Gonzalez, R., 2017. Industrial biomanufacturing: the future of chemical production. *Science* 355.
- Connor, M.R., Cann, A.F., Liao, J.C., 2010. 3-Methyl-1-butanol production in *Escherichia coli*: random mutagenesis and two-phase fermentation. *Appl. Microbiol. Biotechnol.* 86, 1155–1164.
- Conrado, R.J., Gonzalez, R., 2014. Chemistry. Envisioning the bioconversion of methane to liquid fuels. *Science* 343, 621–623.
- Criddle, C.S., 2013. et al. (USA, vol. US20130071890 A1.
- Dellomonaco, C., Clomburg, J.M., Miller, E.N., Gonzalez, R., 2011. Engineered reversal of the β-oxidation cycle for the synthesis of fuels and chemicals. *Nature* 476, 355–359.
- Demidenko, A., Akberdin, I.R., Allemann, M., Allen, E.E., Kalyuzhnaya, M.G., 2016. Fatty acid biosynthesis pathways in *Methylomicrobium buryatense* 5GB1. *Front. Microbiol.* 7, 2167.
- Dittrich, C.R., Bennett, G.N., San, K.Y., 2005. Characterization of the acetate-producing pathways in *Escherichia coli*. *Biotechnol. Prog.* 21, 1062–1067.
- Dunlop, M.J., 2011. Engineering microbes for tolerance to next-generation biofuels. *Biotechnol. Biofuels* 4, 32.
- Espah Borujeni, A., Channarasappa, A.S., Salis, H.M., 2014. Translation rate is controlled by coupled trade-offs between site accessibility, selective RNA unfolding and sliding at upstream standby sites. *Nucleic Acids Res.* 42, 2646–2659.
- Fei, Q., et al., 2014. Bioconversion of natural gas to liquid fuel: opportunities and challenges. *Biotechnol. Adv.* 32, 596–614.
- Fu, Y., Li, Y., Lidstrom, M., 2017. The oxidative TCA cycle operates during methanotrophic growth of the Type I methanotroph *Methylomicrobium buryatense* 5GB1. *Metab. Eng.* 42, 43–51.
- Garg, S., et al., 2016. Microbial production of bi-functional molecules by diversification of the fatty acid pathway. *Metab. Eng.* 35, 9–20.
- Gibson, D.G., et al., 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* 6, 343–345.
- Gilman, A., et al., 2015. Bioreactor performance parameters for an industrially-promising methanotroph *Methylomicrobium buryatense* 5GB1. *Microb. Cell Fact.* 14, 182.
- Gilman, A., et al., 2017. Oxygen-limited metabolism in the methanotroph *Methylomicrobium buryatense* 5GB1C. *PeerJ* 5, e3945.
- Greenhouse Gas Emissions, 2017. Understanding Global Warming Potentials. Environmental Protection Agency.
- Hanson, R.S., Hanson, T.E., 1996. Methanotrophic bacteria. *Microbiol. Rev.* 60, 439–471.
- Hartmanis, M.G., Gatenbeck, S., 1984. Intermediary metabolism in *Clostridium*

- acetobutylicum: levels of enzymes involved in the formation of acetate and butyrate. *Appl. Environ. Microbiol.* 47, 1277–1283.
- Hartmanis, M.G.N., Gatenbeck, S., 1984. Intermediary metabolism in *Clostridium acetobutylicum* - levels of enzymes involved in the formation of acetate and butyrate. *Appl. Environ. Microbiol.* 47, 1277–1283.
- Haynes, C.A., Gonzalez, R., 2014. Rethinking biological activation of methane and conversion to liquid fuels. *Nat. Chem. Biol.* 10, 331–339.
- Henard, C.A., et al., 2016. Bioconversion of methane to lactate by an obligate methanotrophic bacterium. *Sci. Rep.* 6, 21585.
- Henard, C.A., Freed, E.F., Guarnieri, M.T., 2015. Phosphoketolase pathway engineering for carbon-efficient biocatalysis. *Curr. Opin. Biotechnol.* 36, 183–188.
- Henard, C.A., Smith, H.K., Guarnieri, M.T., 2017. Phosphoketolase overexpression increases biomass and lipid yield from methane in an obligate methanotrophic biocatalyst. *Metab. Eng.* 41, 152–158.
- Hu, B., et al., 2016. Comprehensive molecular characterization of *Methylobacterium extorquens* AM1 adapted for 1-butanol tolerance. *Biotechnol. Biofuels* 9, 84.
- Hwang, J.W., Choi, Y.B., Park, S., Choi, C.Y., Lee, E.Y., 2007. Development and mathematical modeling of a two-stage reactor system for trichloroethylene degradation using *Methylosinus trichosporium* OB3b. *Biodegradation* 18, 91–101.
- Kaluzhnaya, M., et al., 2001. Taxonomic characterization of new alkaliphilic and alkalitolerant methanotrophs from soda lakes of the Southeastern Transbaikalian region and description of *Methylobacterium buryatense* sp. nov. *Syst. Appl. Microbiol.* 24, 166–176.
- Kalyuzhnaya, M.G., et al., 2013. Highly efficient methane biocatalysis revealed in a methanotrophic bacterium. *Nat. Commun.* 4, 2785.
- Kalyuzhnaya, M.G., Puri, A.W., Lidstrom, M.E., 2015. Metabolic engineering in methanotrophic bacteria. *Metab. Eng.* 29, 142–152.
- Keasling, J.D., 2012. Synthetic biology and the development of tools for metabolic engineering. *Metab. Eng.* 14, 189–195.
- Khmelenina, V.N., et al., 2013. Draft genome sequence of *Methylobacterium buryatense* strain 5G, a haloalkaline-tolerant methanotrophic bacterium. *Genome Announc.* 1.
- Kim, S., Clomburg, J.M., Gonzalez, R., 2015. Synthesis of medium-chain length (C6–C10) fuels and chemicals via  $\beta$ -oxidation reversal in *Escherichia coli*. *J. Ind. Microbiol. Biotechnol.* 42, 465–475.
- Kim, S., Cheong, S., Gonzalez, R., 2016. Engineering *Escherichia coli* for the synthesis of short- and medium-chain  $\alpha,\beta$ -unsaturated carboxylic acids. *Metab. Eng.* 36, 90–98.
- Kumari, S., Tishel, R., Eisenbach, M., Wolfe, A.J., 1995. Cloning, characterization, and functional expression of *acs*, the gene which encodes acetyl coenzyme A synthetase in *Escherichia coli*. *J. Bacteriol.* 177, 2878–2886.
- Lan, E.I., Liao, J.C., 2012. ATP drives direct photosynthetic production of 1-butanol in cyanobacteria. *Proc. Natl. Acad. Sci. USA* 109, 6018–6023.
- Lin, H., Castro, N.M., Bennett, G.N., San, K.Y., 2006. Acetyl-CoA synthetase overexpression in *Escherichia coli* demonstrates more efficient acetate assimilation and lower acetate accumulation: a potential tool in metabolic engineering. *Appl. Microbiol. Biotechnol.* 71, 870–874.
- Mehta, P.K., Ghose, T.K., Mishra, S., 1991. Methanol biosynthesis by covalently immobilized cells of *Methylosinus trichosporium*: batch and continuous studies. *Biotechnol. Bioeng.* 37, 551–556.
- Nowroozi, F.F., et al., 2014. Metabolic pathway optimization using ribosome binding site variants and combinatorial gene assembly. *Appl. Microbiol. Biotechnol.* 98, 1567–1581.
- Okamura, E., Tomita, T., Sawa, R., Nishiyama, M., Kuzuyama, T., 2010. Unprecedented acetoacetyl-coenzyme A synthesizing enzyme of the thiolase superfamily involved in the mevalonate pathway. *Proc. Natl. Acad. Sci. USA* 107, 11265–11270.
- Puri, A.W., et al., 2015. Genetic tools for the industrially promising methanotroph *Methylobacterium buryatense*. *Appl. Environ. Microbiol.* 81, 1775–1781.
- Salis, H.M., Mirsky, E.A., Voigt, C.A., 2009. Automated design of synthetic ribosome binding sites to control protein expression. *Nat. Biotechnol.* 27, 946–950.
- Shams Yazdani, S., Gonzalez, R., 2008. Engineering *Escherichia coli* for the efficient conversion of glycerol to ethanol and co-products. *Metab. Eng.* 10, 340–351.
- Shen, C.R., et al., 2011. Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. *Appl. Environ. Microbiol.* 77, 2905–2915.
- Shiba, Y., Paradise, E.M., Kirby, J., Ro, D.K., Keasling, J.D., 2007. Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for high-level production of isoprenoids. *Metab. Eng.* 9, 160–168.
- Singh, A., Cher Soh, K., Hatzimanikatis, V., Gill, R.T., 2011. Manipulating redox and ATP balancing for improved production of succinate in *E. coli*. *Metab. Eng.* 13, 76–81.
- Smanski, M.J., et al., 2014. Functional optimization of gene clusters by combinatorial design and assembly. *Nat. Biotechnol.* 32, 1241–1249.
- Stephanopoulos, G., 2002. Metabolic engineering by genome shuffling. *Nat. Biotechnol.* 20, 666–668.
- Strong, P.J., Xie, S., Clarke, W.P., 2015. Methane as a resource: can the methanotrophs add value? *Environ. Sci. Technol.* 49, 4001–4018.
- Strong, P.J., Kalyuzhnaya, M., Silverman, J., Clarke, W.P., 2016. A methanotroph-based biorefinery: potential scenarios for generating multiple products from a single fermentation. *Bioresour. Technol.* 215, 314–323.
- Tollefson, J., 2016. 'Flaring' wastes 3.5% of world's natural gas. *Nature*.
- de la Torre, A., et al., 2015. Genome-scale metabolic reconstructions and theoretical investigation of methane conversion in *Methylobacterium buryatense* strain 5G(B1). *Microb. Cell Fact.* 14, 188.
- U.S. Natural Gas Vented and Flared, 2017. U.S. Department of Energy, Washington, DC.
- Vick, J.E., et al., 2015. *Escherichia coli* enoyl-acyl carrier protein reductase (FabI) supports efficient operation of a functional reversal of  $\beta$ -oxidation cycle. *Appl. Environ. Microbiol.* 81, 1406–1416.
- Whittenbury, R., Phillips, K.C., Wilkinson, J.F., 1970. Enrichment, isolation and some properties of methane-utilizing bacteria. *J. Gen. Microbiol.* 61, 205–218.
- Yan, X., Chu, F., Puri, A.W., Fu, Y., Lidstrom, M.E., 2016. Electroporation-based genetic manipulation in type I methanotrophs. *Appl. Environ. Microbiol.* 82, 2062–2069.
- Ye, R.W., Kelly, K., 2012. Construction of carotenoid biosynthetic pathways through chromosomal integration in methane-utilizing bacterium *Methylobacterium* sp. strain 16a. *Methods Mol. Biol.* 892, 185–195.
- Zhang, B., et al., 2015. Ribosome binding site libraries and pathway modules for shikimic acid synthesis with *Corynebacterium glutamicum*. *Microb. Cell Fact.* 14, 71.